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Cytotoxic effects of ibuprofen on cervical cancer (Hela) cells through induction of nitric oxide synthase2 (iNOS) gene expression

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Abstract: NSAIDs have been reported to have anticancer effects against certain types of cancer, however, the mechanism behind NSAIDs action on cancer cells is unclear in many cases. The aim of this study was to investigate the cytotoxic effects of ibuprofen on cervical cancer (Hela) cells and evaluation of nitric oxide synthase2 (iNOS) gene expression level. In this *in vitro* study, the cells were divided into control (untreated) group and groups treated with 0.01, 0.1, 1, 2.5, 5, and 10 mg/mL of ibuprofen for 24 and 48 hours. MTT assay method was used to determine the cytotoxic effects of ibuprofen on Hela cells. The Griess method was used to quantify NOS activity, and iNOS gene expression level was evaluated using quantitative RT-PCR. The data were analyzed using ANOVA and Student's t-test. Ibuprofen had cytotoxic effects on Hela cells in a dose dependent manner. The cytotoxic dose of ibuprofen significantly increased the NOS activity and iNOS gene expression level. Our findings indicated that the cytotoxic effects of ibuprofen on cervical cancer cells is partly mediated by increased activation of NOS.

Keywords: Hela cell line, Ibuprofen, Nitric oxide synthase

1 Introduction

In 2018, there were over 311000 cervical cancer fatalities and 570000 new instances of the

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illness. After breast cancer (2.1 million cases), colorectal cancer (0.8 million), and lung cancer (0.7 million), cervical cancer was the fourth most frequent malignancy in women. With rates ranging from less than 2 to 75 per 100000 women, the estimated age-standardized incidence of cervical cancer was 13.1 per 100000 women worldwide. In eastern, western, middle, and southern Africa, cervical cancer accounted for the majority of cancer-related deaths among female patients. Eswatini was found to have the highest incidence, with about 65% of women developing cervical cancer before age 75. With 106 000 cases in China and 97000 cases in India, as well as 48000 deaths in China and 60000 deaths in India, China and India collectively contributed more than a third of the global cervical burden. Estimates for 2018 show that 33000 cases of cervical cancer and 15000 deaths from the disease occurred in Europe (Arbyn et al., 2020). The creation of current treatment agents and research into the most effective and innovative therapeutic modalities are the results of the exponential rise of this devastating disease and the fight against cancer. Exploring novel drugs that may be utilized to treat aggressive tumors and have no or fewer negative side effects has become a top priority. Over the last few decades, using and creating new anticancer agents, such as synthetic or natural non-cytotoxic nutrients, has drawn a lot of interest (Hussain et al., 2011). The justification for non-steroidal anti-inflammatory medicines (NSAIDs) is strong in this regard. Numerous epidemiological research revealed that NSAIDs may lower the risk of developing (Zhou et al., 2014). Both selective and non-selective NSAIDs have been shown to protect against and remarkably stop chemically induced carcinogenesis of epithelial tumors by various reliable studies (Collet et al., 1999; Harris et al., 2005). Since NSAIDs have anti-inflammatory, antipyretic, and analgesic characteristics, they may be used in clinical settings (Kim et al., 2003).

Overexpression of the inducible cyclooxygenase-2 (COX-2) gene and inhibition of prostaglandin (PG) biosynthesis are components of the carcinogenesis pathway in many cancers (Harris et al., 2005). NSAIDs reduce prostaglandin production by inhibiting cyclooxygenase (Zhou et al., 2014). Arachidonic acid and ceramide production are involved and stimulated by NSAIDs, which are crucial for the start of apoptosis (Kim et al., 2003). NSAIDs exert their anticancer effects either on the tumors themselves or on the tumor microenvironment by preventing angiogenesis, reducing migration, inhibiting proliferation, and overriding the resistance to apoptosis (De Groot et al., 2007; Rayburn et al., 2009).

Under normal physiological circumstances, the enzyme nitric oxide synthase (NOS) converts L-arginine to L-citrulline to produce NO in the cells. There are three isoforms of NOS: neuronal NO synthase (nNOS, also known as NOS1), inducible NO synthase (iNOS or NOS2), and endothelial NO synthase (eNOS or NOS3) (Geller and Billiar, 1998; Alderton et al., 2001). It is now clear that NO has significant impact on cancer, including modulating apoptosis and metastasis, DNA damage, oncogene activation, tumor suppressor gene suppression, and many stages of carcinogenesis (Wink et al., 1998; Lala and Orucevic, 1998; Thomsen et al., 1995; Xie et al., 1995). It has been demonstrated that NO is a component of the actions of stromal cells, including the immune system and vascular tissue cells, which are part of the tumor microenvironment. Overall, the impact of NO is influenced by iNOS expression levels, timing and duration of NO delivery, the microenvironment, the genetic background, and cell type (Zhang et al., 2007). High levels of NO is associated with cancer cell death (Ridnour et al., 2005). Although studies have shown that ibuprofen has anticancer effects against certain types of

cancer, few studies have been carried out on the effects of ibuprofen on cervical cancer cells. In addition, the association of ibuprofen with NOS activity in cervical cancer cells is unclear. The aim of this *in vitro* study was to investigate the cytotoxic effects of ibuprofen on cervical cancer (Hela) cells and evaluation of nitric oxide synthase2 (iNOS) gene expression and NOS activity level.

2 Materials and Methods

2. 1. Ibuprofen

Ibuprofen was purchased from Aburaihan Pharmaceutical Company as pure powder and a 200 mM stock solution was prepared in DMSO and stored at -20°C until the use.

2. 2. Cell culture

The HeLa cell line was purchased from the National Cell Bank of Iran (NCBI). The samples were frozen in a nitrogen tank. Cells were cultured in DMEM (Dulbecco's Modified Eagle Medium; SIGMA, USA) with 10% fetal bovine serum (FBS) and 1% antibiotic (gentamicin) according to method used in previous experiments (Lucey et al., 2009). Cells were incubated in a humidified atmosphere with 5% CO2 in a 37°C incubator. PBS and trypsin-EDTA were used to wash the cultured cells with 70-80% confluence and separate the cells from the flask, respectively. A culture medium containing 10% FBS was added to neutralize excess trypsin-EDTA activity. The cell suspension was centrifuged, and the cell pellet was suspended in a fresh culture medium and prepared for use in subsequent experiments.

2. 3. Cell viability assay

MTT assay method was used to determine the cell viability (Marks et al., 1992). 1×10^4 cells were cultured in each well of the 96-well plate and plate was placed in a 37°C incubator for 24 hours until 60% of the wells were filled. The cells were treated with ibuprofen (0.01, 0.1, 1, 2.5, 5, 10 mg/mL) for 24, and 48 hours. 100 λ of MTT solution (0.05 mg/well) was added to the wells in the dark. The plate was placed in the incubator for 4 hours, then the supernatant of the wells was removed and 100 λ of isopropanol was added to the wells. Finally, the intensity of the resulting color was read by a microplate reader (DNM-9602G) at a wavelength of 570 nm. Cell viability was calculated as the percentage of viable cells in the total population, and ibuprofen inhibitory concentration (IC50) was calculated.

2. 5. RNA extraction and quantitative RT-PCR

Following the manufacturer's instructions, total RNAs were extracted using a high-purity RNA extraction kit (Takara, Japan) and then reverse transcribed into cDNA. Then, quantitative real-time PCR (RT-PCR) was used to examine GAPDH and iNOS expression levels. Oligo Primer Analysis Software version 7 was used to build the primers (table 1) and then blasted by the NCBI database. Gene expression levels were determined using the $2^{-\Delta\Delta CT}$ method and normalized to the GAPDH loading control (Bustin, 2000).

Table I. Primer sequences used in qRT-PCR

Gene	Primer Sequences
iNOS	Forward: 5′- GTGCCCTGCTTTGTGCG-3′
	Reverse: 5′- TCCTCCTGGTAGATGTGGTCCT-3′
GAPDH	Forward: 5′- CCCACTCCTCCACCTTTGAC-3′
	Reverse: 5′- CATACCAGGAAATGAGCTTGACAA-3′

2. 6. NOS activity assay

Based on the IC50 determined of drug toxicity in the MTT assay test, which was 2.5 mg/mL, cells were treated with 1, 2.5, and 5 mg/mL for 24 and 48 hours. RPMI culture medium was used for NOS activity assay. The Griess method was used to quantify NOS activity (Goshi et al., 2019).

2. 7. Statistical analysis

Data were analyzed using SPSS 18 by one-way analysis of variance (ANOVA) and student's t-test. All data were expressed as the mean ± standard deviation (S.D.) and $p<0.05$ was considered significant.

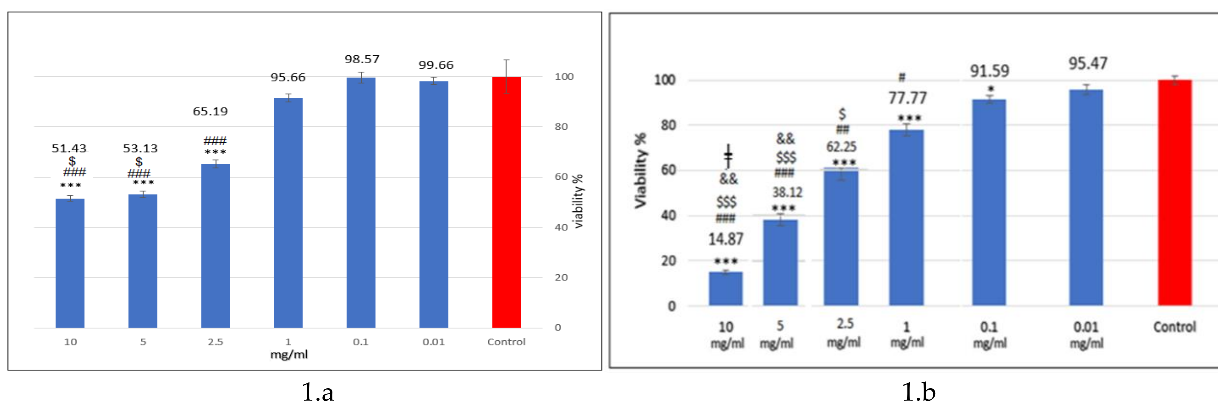
3 Results and Discussions

3. 1. IC50 of Ibuprofen

The curve's data was obtained by plotting the viability of cervical cancer cells (Hela) (%) against the concentration of ibuprofen during 24 and 48 hours of treatment. The IC50 value obtained for the cytotoxic dose of ibuprofen on Hela cells was 2.5 mg/ml.

3. 2. The cytotoxic effects of ibuprofen on Hela cells

Treatment of Hela cells with 0.01, 0.1, and 1 mg/mL of ibuprofen had no significant effect on the cell viability 24 hours after treatment compared to control group, however, treatment of Hela cells with 2.5, 5, and 10 mg/mL of ibuprofen caused a significant decrease in cell viability compared to control group ($p<0.001$) (Figure 1a). 48 hours after treatment, 0.01 mg/mL of ibuprofen had no significant effect on Hela cells viability, however, 0.1, 1, 2.5, 5, and 10 mg/mL of ibuprofen caused a significant decrease in cell viability compared to control group (Figure 2b).



1.a 1.b

Figure 1. The effect of ibuprofen on Hela cells 24 hours (1.a) and 48 hours (1.b) after treatment. *, #, $, & and ǂ indicate a significant difference compared to control group (*: p<0.05; ***: p<0.001, ###: p<0.001, $: p<0.05, $: p<0.05, $$$: p<0.001, &&: p<0.01, and ǂ: p< 0.05)

Treatment of Hela cells with 0.1, 1, 2.5, 5, and 10 mg/ml of ibuprofen caused a more significant decrease in the viability of Hela cells 48 hours after treatment compared to the corresponding groups 24 hours after treatment (Figure 2).

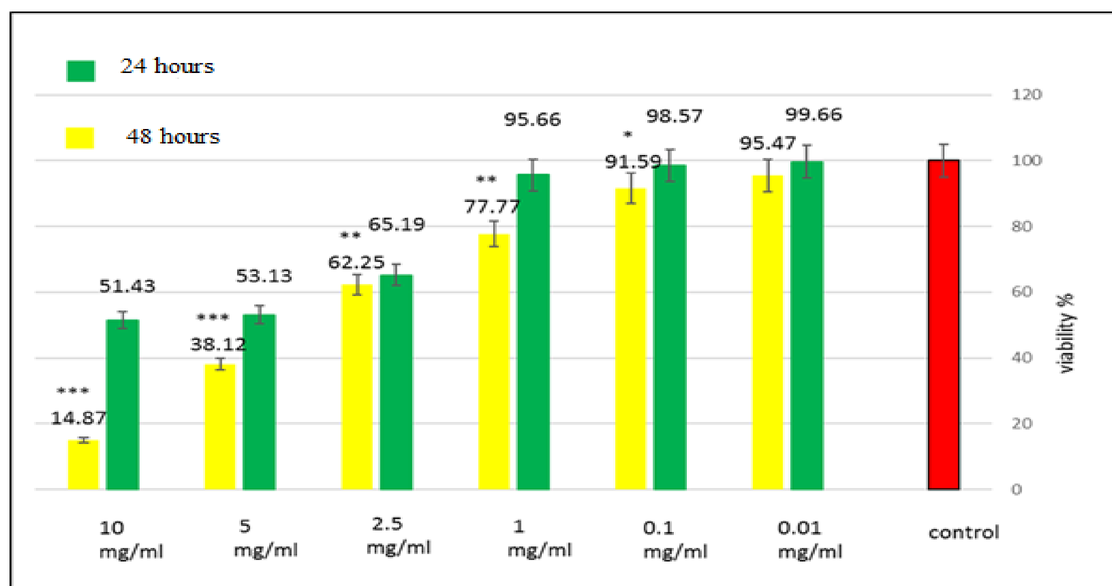
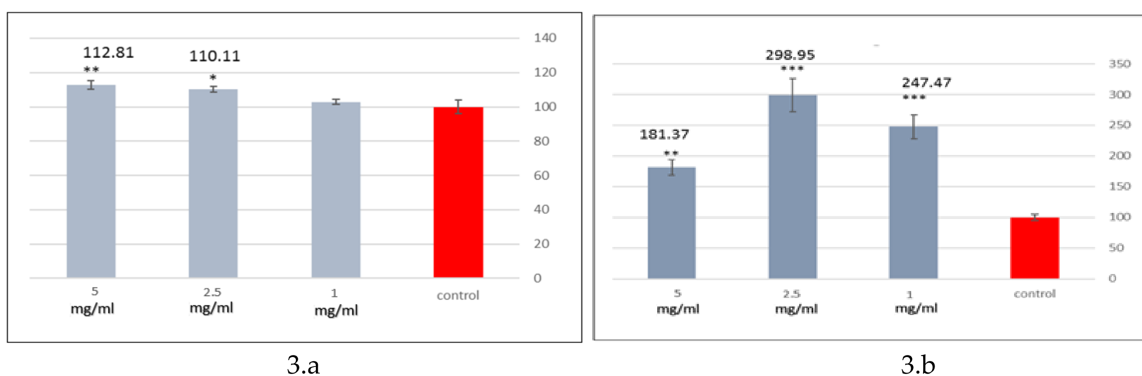


Figure 2. Comparison of the effects of different concentrations of ibuprofen on Hela cells viability during 24 and 48 hours after treatment. * indicates significant difference (*: p<0.05, **: p<0.01 and ***: p<0.001)

3. 3. The effect of ibuprofen on nitric oxide synthase activity level in Hela cells

Treatment of Hela cells with 2.5 and 5 mg/mL of ibuprofen caused a significant increase in the activity level of nitric oxide synthase compared to control group 24 and 48 hours after treatment (Figure 3). Although 1 mg/mL of ibuprofen could significantly increase the nitric oxide synthase activity level 48 hours after treatment, it could not change significantly the enzyme activity level 24 hours after treatment (Figure 3).



3.a 3.b

Figure 3. The effect of different concentrations of ibuprofen on nitric oxide synthase activity level in Hela cells 24 hours (3.a) and 48 hours (3.b) after treatment. * indicates a significant difference compared to control group (*: p<0.05, **: p<0.01 and ***: p<0.01).

3. 4. The effects of ibuprofen on the relative expression of iNOS gene in Hela cells

Treatment of Hela cells with 2.5 mg/mL of ibuprofen led to significant increase in expression level of nitric oxide synthase gene compared to control group (Figure 4).

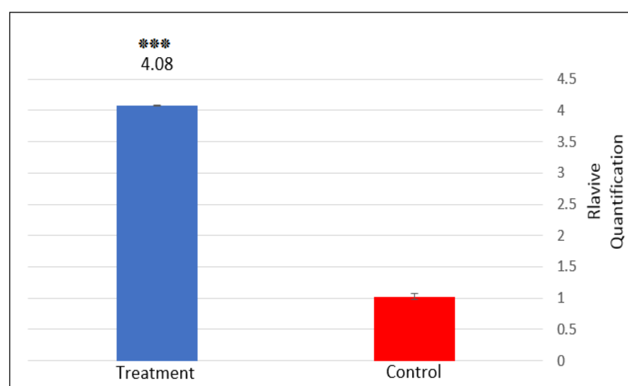


Figure 4. The effect of ibuprofen on the relative expression level of iNOS gene in Hela cells 48 hours after treatment. * indicates a significant difference compared to the control group (***:p<0.001).

Our findings indicated that ibuprofen has *in vitro* cytotoxic effects on cervical cancer cells in a dose dependent manner. The cytotoxic effect of ibuprofen on cervical cancer cells is partly mediated by increased iNOS gene expression and increased NOS activity level. In consistent with our findings, Kwak et al. have shown in a study that the death of tumor cells can be induced by inducing iNOS in cancer cells (Kwak et al., 2000). Nitric oxide enzyme was studied by Sun et al. in 2013 for its impact on esophageal cancer, and the findings showed that this enzyme promoted apoptosis in esophageal cancer cells (Sun et al., 2013). Additionally, they found that the nitric oxide enzyme causes cancer cells to progress toward apoptosis by altering the activity of the p53 gene and boosting the p53 gene's expression (Muntane et al., 2013). In terms of the possible mechanism of the effect of ibuprofen on cervical cancer cells, it can be assumed that after binding to its receptor, ibuprofen can induce the expression of iNOS gene on

the one hand and increase the activity of the nitric oxide synthase enzyme on the other hand by activating the signaling pathways. And this probably causes the induction of apoptosis in cervical cancer cells, which itself leads to cell death and stops the proliferation of cervical cancer cells.

4 Conclusion

The results of this research showed that ibuprofen induces cytotoxic effects in cervical cancer (Hela) cells *in vitro* in a dose- and time- dependent manner. The ibuprofen cytotoxic effects on cervical cancer cells is partly mediated by increased iNOS gene expression level and increased NOS activity level.

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Conflict of interests

The authors state that there are no conflicts of interest regarding the publication of this paper.

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